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Isolation, identification, and synthesis of 2-carboxyarabinitol 1-phosphate, a diurnal regulator of ribulose-bisphosphate carboxylase activity

(photosynthesis/CO_2 fixation/enzyme regulation/enzyme inhibitor/phosphate ester)

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ABSTRACT **The diurnal change in activity of ribulose 1,5-bisphosphate (Rbu-1,5-P_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39] of leaves of *Phaseolus vulgaris* is regulated (in part) by mechanisms that control the level of an endogenous inhibitor that binds tightly to the activated (carbamoylated) form of Rbu-1,5-P_2 carboxylase. This inhibitor was extracted from leaves and copurified with the Rbu-1,5-P_2 carboxylase of the leaves. Further purification by ion-exchange chromatography, adsorption to purified Rbu-1,5-P_2 carboxylase, barium precipitation, and HPLC separation yielded a phosphorylated compound that was a strong inhibitor of Rbu-1,5-P_2 carboxylase. The compound was analyzed by GC/MS, ^{13}C NMR, and ^1H NMR and shown to be 2-carboxyarabinitol 1-phosphate [(2-*C*-phosphohydroxymethyl)-D-ribonic acid]. Verification of structure was obtained by comparison of the inhibitory activity of the isolated compound with that of 2-carboxy-D-arabinitol 1-phosphate synthesized *in vitro*. This compound (but not 2-carboxy-D-arabinitol 5-phosphate) inhibited Rbu-1,5-P_2 carboxylase in a way that was kinetically identical to that of the isolated, naturally occurring compound. The structure of the isolated compound differs from the Rbu-1,5-P_2 carboxylase transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate only by the lack of the C-5 phosphate group. This difference results in a higher binding constant for the monophosphate (K_d = 32 nM) compared with the bisphosphate (K_d < 10 pM). The less tightly bound compound acts in a light-dependent, reversible regulation of Rbu-1,5-P_2 carboxylase activity *in vivo*.**

The activity of ribulose 1,5-bisphosphate (Rbu-1,5-P_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39] of *Phaseolus vulgaris* (garden bean) and several other species (1–5) is influenced by mechanisms that control the *in vivo* concentration of an inhibitor that binds tightly to the active site of the enzyme, thus rendering it catalytically inactive. This mechanism appears to function together with the reversible formation of the active (carbaminated) site in regulating the activity of Rbu-1,5-P_2 carboxylase *in vivo* (6). Previous work has shown that the inhibitor is a phosphorylated compound (1, 2), that it binds to the activated (carbaminated) form of the enzyme yielding a stable enzyme–inhibitor complex (2), that it is compartmented in the chloroplast (2), and that its concentration varies under natural conditions according to a diurnal pattern such that enzymatic capacity for CO_2 fixation is low at night and maximal at midday (2, 6).

We report the structural identity of the inhibitor present in leaves of *P. vulgaris*, the synthesis of that compound *in vitro*, and some kinetic parameters associated with its interaction with Rbu-1,5-P_2 carboxylase. A preliminary report of this work has been presented (7).

MATERIALS AND METHODS

Plant Material. *P. vulgaris* (cv. Blue Lake) plants were grown under natural illumination in a greenhouse in a flowing nutrient system containing 0.5× Hoagland's solution (8). Temperature was kept at 20–25°C during the day and >16°C at night. Supplemental illumination was provided during the winter months.

Extraction of Inhibitor. Leaf material (typically 1 kg per batch) of *P. vulgaris* plants kept in darkness overnight was frozen in liquid nitrogen and pulverized in a mortar and pestle. The powder was mixed with 1.0 liter of 50 mM Tris, pH 8.0/20 mM $NaHCO_3$/60 mM $MgCl_2$/10 mM mercaptoethanol/5 mM sodium ascorbate/1 mM EDTA and then homogenized using a Waring blender and a Polytron homogenizer (Brinkman); the homogenate was filtered and centrifuged. Rbu-1,5-P_2 carboxylase was then precipitated by adding a sufficient volume of 60% (wt/vol) polyethyleneglycol (P-3640, Sigma) to yield a final concentration of 18%. This solution was kept at 0°C for 1 hr with stirring, and the precipitated Rbu-1,5-P_2 carboxylase was collected by centrifugation at 13,000 × *g* for 30 min. The Rbu-1,5-P_2 carboxylase with bound inhibitor was dissolved in a minimal volume of water; $HClO_4$ was added to 0.45 M, precipitating the Rbu-1,5-P_2 carboxylase and releasing the inhibitor. The suspension was centrifuged at 15,000 × *g* for 10 min, and ClO_4^- was removed from the supernatant by adding KOH to pH 7 followed by centrifugation at 5000 × *g* for 10 min. The supernatant was passed through a column of Dowex 50 (H^+ form), and the column was washed with 2-column volumes of H_2O. The effluent was then applied to a 0.8 × 20-cm column of Dowex 1 (formate form) that bound the inhibitor, and the latter was eluted with a linear gradient of formic acid (0–8 M). These fractions were assayed (see *Results*), combined, and dried in a rotary evaporator under vacuum. The residue was next dissolved in water, brought to pH 10, and kept at pH > 9 with careful additions of KOH until cessation of base uptake. The inhibitor was then precipitated at pH 8.5 by the addition of 20 mM $BaCl_2$ and 2 volumes of 95% ethanol. The precipitate was collected and washed with 50% ethanol (vol/vol). It was then dissolved in water with Dowex 50 (H^+ form), and pH was adjusted to between 7 and 8 for storage.

Rbu-1,5-P_2 Carboxylase. Purified spinach Rbu-1,5-P_2 carboxylase was used for all kinetic studies of the inhibitor. This

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Abbreviations: CA-1,5-P_2, 2-carboxyarabinitol 1,5-bisphosphate; CA-1-*P*, 2-carboxyarabinitol 1-phosphate; Rbu-1,5-P_2, ribulose 1,5-bisphosphate; Me_3Si, trimethylsilyl (substituent group).

was prepared from market spinach, with the method described by Hall and Tolbert (9). The concentration of Rbu-1,5-P_2 carboxylase was determined essentially as described by Seemann *et al.* (10) by radiolabeling with 2-[^{14}C]carboxyarabinitol 1,5-bisphosphate {[^{14}C](CA-1,5-P_2); 2146 GBq/mol} and immunoprecipitation with rabbit antisera to spinach Rbu-1,5-P_2 carboxylase. Activity assays were conducted at 25°C in 0.5 ml of 100 mM Bicine (*N,N*-bis(2-hydroxethyl)-glycine)/10 mM $MgCl_2$/0.1 mM EDTA/5 mM dithiothreitol/10 mM [^{14}C]$NaHCO_3$ (18.5 GBq/mol) containing 0.4–0.7 μM Rbu-1,5-P_2 carboxylase active sites. The reaction was started by adding Rbu-1,5-P_2 to a final concentration of 0.4 mM. Assays were stopped after 1 min by the addition of 0.1 ml of 2 M HCl, and the acid-stable radioactivity was determined by scintillation counting. When assaying for the inhibitor, an aliquot of the inhibitor-containing preparation was added to the complete assay mixture (enzyme but without Rbu-1,5-P_2); the mixture was incubated for at least 30 min, and then catalytic activity was assayed by adding Rbu-1,5-P_2. Assays of the dissociation kinetics of the inhibitor–enzyme complex were conducted in 100 μl of the assay buffer containing 60 μM Rbu-1,5-P_2 carboxylase and inhibitor. After 30 min of incubation, alkaline phosphatase (≈10 units) was added, and 5-μl aliquots were withdrawn at intervals and Rbu-1,5-P_2 carboxylase activity was assayed by adding medium containing Rbu-1,5-P_2. Dissociation of the enzyme–inhibitor complex releases free enzyme (increasing catalytic activity) and free inhibitor that is degraded by the phosphatase; the quantity of phosphatase is not rate-limiting.

HPLC Separations. Compounds were separated by anion-exchange chromatography and detected with the phosphate-specific system described by Meek and Nicoletti (11).

GC/MS. The inhibitor was incubated overnight in 50 mM NH_4HCO_3, pH 9.5, with several units of alkaline phosphatase. This reaction mixture was treated with Dowex 50 (H^+ form), and the resin was removed by filtration. The filtrate plus washings was reduced to dryness under vacuum. Dried residue was silylated with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide, and aliquots of the silylated material were injected onto a 0.53 mm × 30 m DB-1 column (J & W Scientific, Inc., Rancho Cordova, CA) with a temperature program from 60°C at 8°C·min^{-1}. Mass spectra were recorded at 70 eV. Authentic 2-carboxyarabinitol was prepared by alkaline phosphatase treatment of CA-1,5-P_2.

NMR Studies. ^{1}H-decoupled ^{13}C NMR spectra were collected at 75.6 MHz, and ^{1}H NMR spectra were collected at 360 MHz using Nicolet (Madison, WI) instruments and standard protocols.

RESULTS

Quantitation of the Inhibitor. The concentration of inhibitor was determined by analysis of the inhibition of Rbu-1,5-P_2 carboxylase activity in assays where purified spinach Rbu-1,5-P_2 carboxylase (of known concentration) was incubated with a range of inhibitor dilutions. [See Fig. 4*A*; a plot of the ratio of the inhibited rate (v) to the control rate (v_0) against the concentration of added inhibitor.] An enzyme–inhibitor complex (EI) is formed by reaction of the inhibitor (I) with the enzyme (E) according to the equilibrium, E + I ⇄ EI established during the 30-min incubation. Because EI does not appreciably dissociate during the 1-min time course of the assay, the assay with Rbu-1,5-P_2 measures, $v = k_{cat}[E]$, where [E] is the concentration of free enzyme at equilibrium, and because $E = E_{tot} - EI$, then

$$v/v_0 = (1 - EI)/E_{tot}, \quad [1]$$

where v and v_0 are the inhibited and control rates of catalysis, k_{cat} is the catalytic constant (that cancels), and E_{tot} is the total concentration of enzyme active sites (measured by [^{14}C]CA-1,5-P_2 binding). The equilibrium concentration of EI can be related to the total concentration of inhibitor (I_{tot}) and the dissociation constant (K_d) by the following quadratic equation,

$$EI = \frac{(I_{tot} + E_{tot} + K_d) - [(I_{tot} + E_{tot} + K_d)^2 - 4I_{tot}E_{tot}]^{1/2}}{2} \quad [2]$$

Combining Eq. **1** and Eq. **2** gives an expression for v/v_0 with two unknowns, I_{tot} and K_d. A computer program (12) was used to obtain the best fit for these parameters to experimental data sets with five or more values of I_{tot} giving a wide range of v/v_0. Estimates of inhibitor concentration obtained in this way were generally 30–40% higher than those obtained by the approximation used in ref. 2.

Purification of the Inhibitor. The initial preparations of inhibitor contained other phosphorylated compounds and some UV absorbing contaminants. Advantage was taken of the tight and specific binding of inhibitor to activated Rbu-1,5-P_2 carboxylase to purify the preparations further. A 10% molar excess of inhibitor was added to purified spinach Rbu-1,5-P_2 carboxylase (11.5 μmol) in 50 ml of 0.1 M Tris acetate/10 mM $MgCl_2$/10 mM $NaHCO_3$. The solution was placed in an ultrafiltration cell (Amicon PM-30 membrane) and dialysis-filtered with 400 ml of buffer. Any low-molecular-weight contaminants that may bind to the enzyme (but with higher K_d than that of the inhibitor) should be displaced by the inhibitor excess; these potential contaminants were removed by the diafiltration treatment. The enzyme solution was then acidified (pH < 4.5) by addition of acetic acid, and the precipitated Rbu-1,5-P_2 carboxylase was removed by centrifugation. Treatment with Dowex 50 (H^+ form) removed cations, and the acetic acid solution was evaporated *in vacuo*, yielding 9.7 μmol of inhibitor.

Further purification was achieved by HPLC chromatography on a 0.46 × 10-cm column of Aminex A-27 (Bio-Rad) developed with 10 mM Hepes (pH 7.4) and 0.08 M Na_2SO_4. The column effluent flowed through a UV detector (260 nm) and could be directed to a fraction collector or to a system for the detection of phosphorylated compounds (11). For phosphate detection the effluent was passed through a column of immobilized alkaline phosphatase to hydrolyze organic phosphate and then was mixed with HNO_3 plus ammonium molybdate to produce the phosphomolybdate complex that was detected at 380 nm. For inhibitor detection fractions were collected and aliquots were assayed for inhibitor. Two phosphate-containing peaks were separated from the single peak of UV-absorbing material, and inhibitor activity coincided with the second peak (Fig. 1). This peak was collected from several HPLC separations and combined; the Hepes and Na_2SO_4 were separated from the target compound on a Dowex 1 (HCO_3^- form) column with a linear gradient of ammonium bicarbonate, and the active fractions were treated with Dowex 50 (H^+ form) and concentrated *in vacuo*.

Characterization of the Inhibitor. Previous work (1, 2) showed that the inhibitor was a phosphorylated compound, and the HPLC-purified material contained 1 mol of organic phosphate per mol of inhibitor, confirming the inference of Servaities (1) that the inhibitor is a monophosphate ester. Titration of the purified material (data not shown) indicated the presence of an additional acidic group with a pK_a near 4.5. When taken to dryness under acidic conditions *in vacuo*, the inhibitor formed a derivative with lower inhibitory activity. High activity could be restored by saponifying the preparations at a pH > 10 for 1 hr at 25°C. Monitoring pH after the addition of base showed a slow consumption of base with eventual consumption of an amount of base approximately

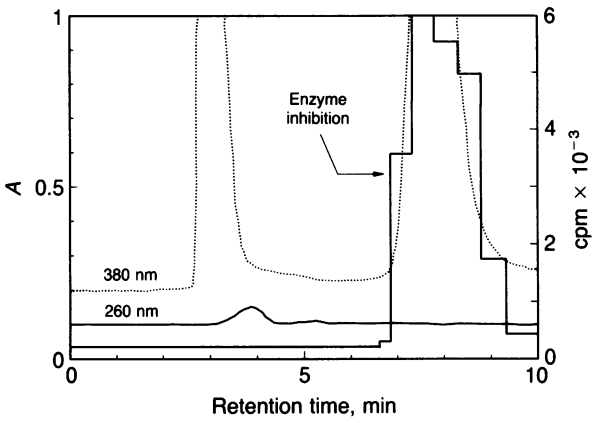


FIG. 1. HPLC of an inhibitor preparation. Solid line labeled 260 represents absorbance before and dotted line labeled 380 after development of the phosphomolybdate complex. Enzyme inhibition is plotted as $v_0 - v$ (in cpm × 10^{-3}) of 5-μl aliquots of fractions of the effluent (collected in a separate run).

equal to the amount of inhibitor present. Such observations suggested that the inhibitor forms a lactone.

GC/MS. To facilitate analysis, the highly purified inhibitor from the HPLC separation was dephosphorylated and silylated. Gas chromatography of the silylated mixture revealed three major peaks (data not shown): A, B, and C with relative areas of 1.0:0.52:0.47 at 11, 17.5, and 21 min, respectively. Peak A was identified as trimethylsilyl($Me_3Si)_3PO_4$, and peaks B and C have mass spectra that are essentially identical to spectra of authentic ($Me_3Si)_6$-2-*C*-hydroxymethylribonic acid (the Me_3Si derivative of 2-carboxyarabinitol) in its free acid form (Fig. 2) and its 1,4-lactone form (data not shown). The three peaks correspond to the expected derivatives of a single compound, and the similarity between the spectra of the inhibitor and standard is obvious. The mass spectral evidence clearly identifies the inhibitor as the phosphate ester of a 2-carboxypentitol. However, these data are insufficient to establish the configuration of the asymmetric carbons or the location of the phosphate atom.

NMR Analysis. A ^{1}H-decoupled ^{13}C NMR spectrum of the monotris(hydroxymethyl)aminomethane salt of the lactone form of purified inhibitor (Fig. 3) shows resonances for each of the six carbon atoms expected for a 2-carboxypentitol derivative. The observed spectrum is similar in all respects to authentic CA-1,5-P_2 lactone (13), excepting that coupling from only one phosphorous atom is evident. The chemical shift assignments (see Fig. 3) were made by comparison with standards selectively enriched with ^{13}C (13) and on the predictable coupling pattern of phosphorous to carbon (14, 15). The doublets marked c and e arise from ^{13}C–^{31}P coupling and indicate that the phosphate ester is esterified to a hydroxymethyl carbon adjacent to a quaternary carbon (e.g., C-1). The hydroxymethyl carbon e is split by two-bond carbon–phosphorous coupling (^2J = 4.3 Hz) and is shifted to 64.0 ppm, similar to the two hydroxymethyl carbons of CA-1,5-P_2 lactone (13). The quaternary carbon (C-2) resonance (c, 74.6 ppm) is split by three-bond carbon–phosphorous coupling (^3J = 10.6 Hz) and is distinguished from the other alcoholic resonances by a narrower line-width and substantially smaller intensity (predictable from the absence of a directly bound proton). The C-4 carbon resonance (b) is shifted to 83.4 ppm due to its involvement in the lactone ring. When the inhibitor is converted to its free acid form by saponification, the C-4 carbon resonance is shifted to 72.0 ppm (at pH > 8.0). By way of comparison, the corresponding carbon in CA-1,5-P_2 lactone shifts from 83.9 to 72.9 ppm upon saponification (J.P., unpublished observation). The chemical shift of the secondary alcoholic carbon (C-3) is particularly diagnostic of the stereochemistry of the lactone ring (13), and its identity with that of CA-1,5-P_2 suggests that the compound has the same stereochemistry.

This expectation was confirmed by comparison of the ^{1}H spectrum of the dephosphorylated inhibitor with the spectra of the four authentic 2-carboxy-D-pentitols (16). The spectra of the authentic compounds (data not shown) were easily distinguishable (from each other), and the spectrum of the dephosphorylated inhibitor conformed with that of 2-carboxyarabinitol. Positioning of the phosphorous atom at C-1 was also confirmed by the ^{1}H NMR spectrum of the inhibitor. The multiplet corresponding to the protons on the carbon

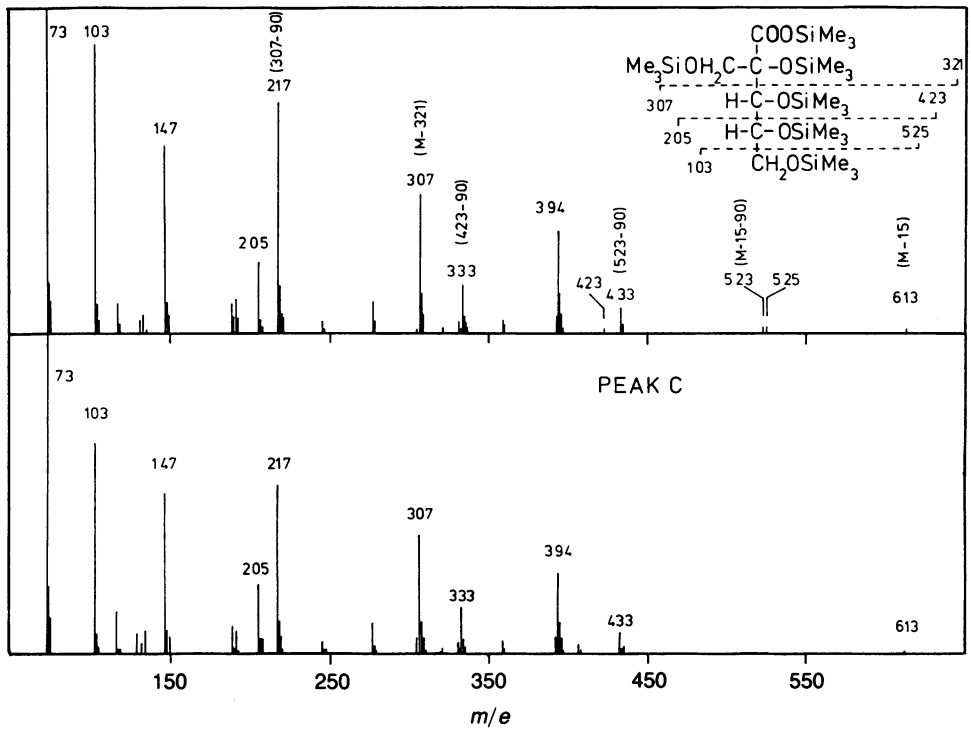


FIG. 2. Comparison of mass spectra of peak C (*Lower*) and authentic Me_3Si_6-2-*C*-hydroxymethyl-D-ribonic acid (identical with Me_3Si_6-2-carboxyarabinitol) (*Upper*). The structural diagram of this compound (*Inset*) shows the masses of major fragments derived from it, and identifiers above peaks relate them to the diagram.

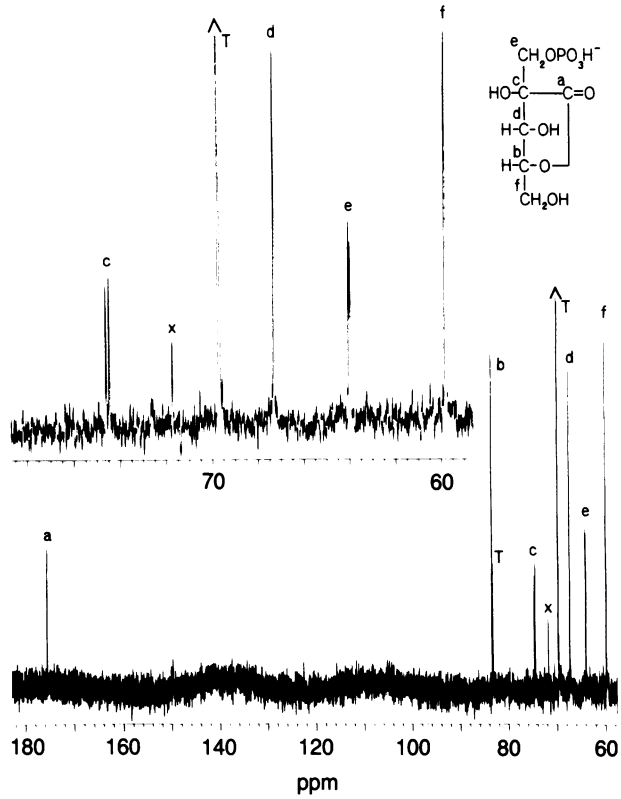


FIG. 3. ^{1}H-decoupled ^{13}C NMR spectrum of the 1,4-lactone form of the monotris(hydroxymethyl)aminomethane salt of the inhibitor. The resonances marked T are from tris(hydroxymethyl)aminomethane. [The resonance marked X is thought to be C-3 of the free acid form of the inhibitor arising from lactone hydrolysis during the course of the NMR experiment (c.f., ref. 13). This is not a contaminant, because spectra taken of the free acid form of the inhibitor contained no extra resonances.] (*Inset*) Expanded view of the resonances showing phosphorous–carbon coupling.

with the phosphorous atom was split only by phosphorous coupling (and not by other protons), indicating the adjacent carbon lacked protons. This would only be possible if the phosphorous atom is at C-1. We therefore conclude that the compound is carboxyarabinitol 1-phosphate (CA-1-*P*)—i.e., (2-*C*-phosphohydroxymethyl)-D-ribonic acid).

Synthesis of the Inhibitor. Given the 1:1 phosphate/enzyme stoichiometry of Rbu-1,5-P_2 carboxylase inhibition by the purified inhibitor, it is most probable that CA-1-*P*, and not some minor undetected contaminant in the preparation, is the compound that causes the inhibition. To verify this and to determine whether it has the D or L configuration, we prepared CA-1-*P* of the D configuration (and related compounds) and measured their effects on Rbu-1,5-P_2 carboxylase.

Reaction of ribulose-5-phosphate with CN^- was used to prepare a mixture of 2-carboxyarabinitol 5-phosphate and its corresponding diastereoisomer 2-carboxyribitol 5-phosphate. The reaction was conducted as in ref. 13, and most of the added $^{14}CN^-$ tracer (88%) was incorporated into acid-stable products. These showed chromatographic behavior like that of the inhibitor on Dowex 1 (formate form). However, this material did not form a stable inhibitor–enzyme complex when incubated with Rbu-1,5-P_2 carboxylase (Fig. 4*A*).

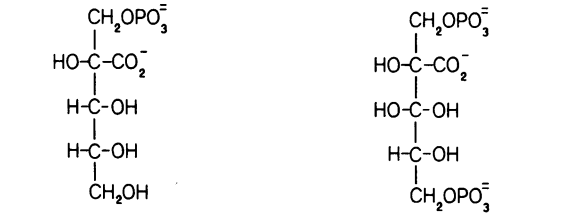
CA-1-*P* was purified from the products of partial phosphatase digestion of CA-1,5-P_2. CA-1,5-P_2 was prepared as described in ref. 13, and a tracer of *p*-nitrophenyl phosphate was added to follow the progress of the phosphatase reaction spectrophotometrically. In pilot experiments CA-1,5-P_2 reacted six to seven times more slowly than did the *p*-nitrophenyl phosphate. Thus, the incubation of CA-1,5-P_2 with alkaline phosphatase (≈2 units, bovine intestinal type from Sigma) was conducted for an interval six times that required to hydrolyze 50% of the *p*-nitrophenyl phosphate in the mixture. The reaction was stopped with $HClO_4$ and neutralized with KOH. Approximately 40% of the esterified phosphate had been released in the incubation. Monophosphates were separated from the free acid and the bisphosphate forms by chromatography on Dowex 1 (formate form). The monophosphate fraction, a strong inhibitor of Rbu-1,5-P_2 carboxylase, was purified by ligation to purified spinach Rbu-1,5-P_2 carboxylase as described for purification of the inhibitor. Because 2-carboxyarabinitol 5-phosphate (the other monophosphate produced from CA-1,5-P_2) apparently has a much higher K_d for binding to Rbu-1,5-P_2 carboxylase (Fig. 4*A*), enzyme-bound material is assumed to be highly enriched in CA-1-*P*.

The CA-1-*P* produced *in vitro* had kinetic properties similar to that of the natural inhibitor (Fig. 4); its binding constant (32 nM) was identical to that of a preparation of natural inhibitor (Fig. 4*A*), and the dissociation rate of the complex formed between the enzyme and the inhibitor or CA-1-*P* was also identical (Fig. 4*B*). CA-1,5-P_2, the starting material for the synthesis, also strongly inhibits Rbu-1,5-P_2 carboxylase, but CA-1,5-P_2 shows much slower kinetics of dissociation (2).

DISCUSSION

We conclude that the naturally occurring inhibitor of Rbu-1,5-P_2 carboxylase that is associated with diurnal variations in Rbu-1,5-P_2 carboxylase activity is the D form of CA-1-*P*. This compound has been concurrently identified by Gutteridge *et al.* (17) as present in potato leaves at light-dependent levels. Thus, light-dependent regulation of Rbu-1,5-P_2 carboxylase in these taxonomically unrelated species is mediated by the same compound. This compound has very close structural similarity to CA-1,5-P_2 and to the six-carbon intermediate formed in the carboxylation of Rbu-1,5-P_2 (Scheme I).

Scheme I



2-Carboxy-arabinitol 1-phosphate

2-Carboxy-3-keto-arabinitol 1,5-bisphosphate (hydrate form)

It is therefore not surprising that the material is a strong inhibitor of Rbu-1,5-P_2 carboxylase. That other compounds of related structure may play a similar role in other organisms should still be considered.

Structural identification of the inhibitor leads to questions about its role in the regulation of chloroplast metabolism and the mechanisms of its synthesis and degradation. Recent work has shown that regulation of the activity of Rbu-1,5-P_2 carboxylase of *P. vulgaris in vivo* involves both modulation of the Mg^{2+}, CO_2-dependent carbamoylation of the active site and modulation of the concentration of CA-1-*P* (6). The CA-1-*P* mechanism seems to be most important in light-dependent regulation of Rbu-1,5-P_2 carboxylase activity,

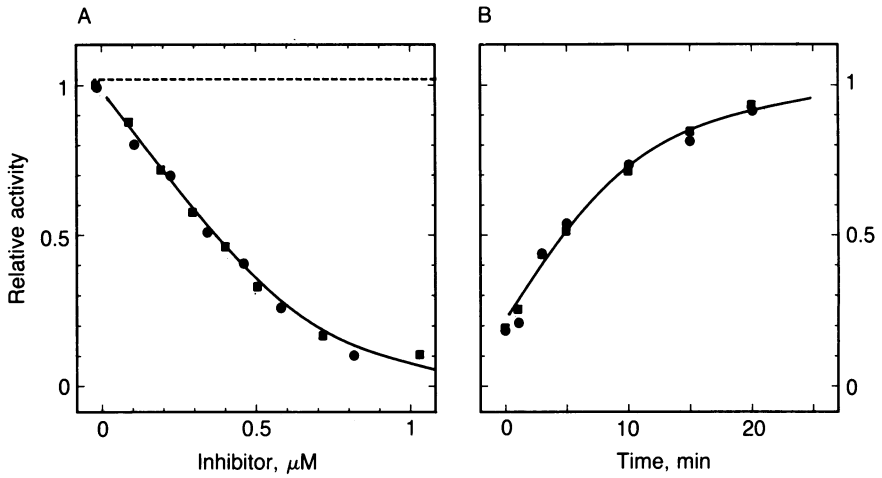


FIG. 4. Kinetic studies of the interaction of inhibitor obtained from *P. vulgaris* and of related compounds synthesized *in vitro* with purified spinach Rbu-1,5-P_2 carboxylase. (*A*) Comparison of the kinetics of inhibition of Rbu-1,5-P_2 carboxylase (0.67 μM) incubated with purified inhibitor (●) and of synthetic preparations of carboxyarabinitol-5-phosphate (----) and CA-1-*P* (■). Rates are plotted as the ratio v/v_0 where v_0 is the control rate. The dashed line for carboxyarabinitol 5-phosphate is a linear regression for seven data points extending to 22 μM. The solid line is from Eq. 1 using K_d = 32 nM. (*B*) Time course of recovery of Rbu-1,5-P_2 carboxylase activity after treating the EI complex with a saturating activity of alkaline phosphatase. Symbols are the same as for *A*, and rates are plotted as the ratio v/v_f, where v_f is the extrapolated final rate. Line is of a first-order process with a rate constant of 0.11 min^{-1} ($t_{1/2}$ = 6.2 min).

whereas carbamination seems to be important in responses to CO_2 and O_2 concentrations (6).

Why there are two separate mechanisms for regulation of the activity of this enzyme is not apparent. One intriguing possibility, however, can be suggested. When in low light or darkness, much of the leaf Rbu-1,5-P_2 carboxylase is in an inactive form. If enzyme activity were reduced by removal of the carbamate at the active site, a binary complex would probably be formed between the decarbaminated enzyme and Rbu-1,5-P_2, which may be present at high concentration even in low light (6). Higher plant Rbu-1,5-P_2 carboxylases bind Rbu-1,5-P_2 tenaciously when in the nonactivated form (K_d of Rbu-1,5-P_2, ≈ 10 nM) (18); this complex is so resistant to activation by CO_2 and Mg^{2+} that >10 hr are required to reach equilibrium activation *in vitro*. (Presumably, activation of this complex is limited by the slow rate of Rbu-1,5-P_2 release.) Recent work has identified a chloroplast protein thought to be required for activation of this binary complex *in vivo* (19). It is possible that the CA-1-*P* mechanism permits regulation while at the same time preventing the formation of the inactive complex with Rbu-1,5-P_2.

Defining the pathway of synthesis presents an interesting problem, because an obvious route via addition of a carboxyl group to Rbu-1,5-P_2, perhaps by reduction of the intermediate 2-carboxy-3-keto-arabinitol 1,5-bisphosphate formed in carboxylation (13, 16), involves the formation of CA-1,5-P_2, which could bind to Rbu-1,5-P_2 carboxylase, permanently inactivating it. The C-5 phosphate group would have to be removed before the compound could function as a regulator of Rbu-1,5-P_2 carboxylase. Another possible route for synthesis would be an aldol condensation of 3-phosphoglyceraldehyde with glyceraldehyde.

Although the mechanisms that degrade this compound *in vivo* are unknown, clearly these mechanisms are somehow linked to light [presumably through photosynthetic electron transport (2)]. Demonstration that the inhibitor can be synthesized from CA-1,5-P_2 suggests the synthesis of labeled material for studies of CA-1-*P* metabolism by chloroplast extracts.

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